# Properties of Photogenerated Tryptophan and Tyrosyl Radicals in Structurally Characterized Proteins Containing Rhenium(I) Tricarbonyl Diimines

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Aromatic amino acid radicals are key intermediates in nucleic acid biosynthesis,[1] DNA repair,[2,3] dioxygen reduction by cytochrome oxidase,[4] water oxidation by PSII,[5] as well as other biological processes.[6−9] In our work on electron tunneling in proteins,[10−12] we have developed laser flash/quench methods that potentially could facilitate the study of such highly reactive radicals.[12−14] To test our methods, we are investigating two structurally characterized proteins, $[Re(CO)_3(L)(H83)]^+AzM^{2+}$ and $[Re(CO)_3(L)(H107)]^+AzM^{2+}$ (L = 1,10-phenanthroline (phen) or 4,7-$Me_2$phen; Az = *Pseudomonas aeruginosa* azurin; M = Cu or Zn).[15,16] Of special interest is that calculations and experiments on the H107 protein show that $Cu^+$ oxidation via electron transfer (ET) through an intervening tyrosine ($Cu^+ \rightarrow Y108^{\bullet/-} \rightarrow Re^{2+}$) is over 2 orders of magnitude faster than optimized ($Cu^+ \rightarrow Re^{2+}$) electron tunneling.[12]

We report that phototriggered irreversible oxidation of $[Re(CO)_3(phen)(H107)]^+AzZn^{2+}$ produces $Y108^{\bullet}$;[17] and that the same method can be employed to generate W48$^{\bullet}$ in the H83 protein (Scheme 1).

The $g = 2.0061$ signal in the EPR spectrum of a frozen solution of irradiated $[Re(CO)_3(phen)(H83)]^+AzZn^{2+}/[Co(NH_3)_5Cl]^{2+}$ (Figure 1A)[18] is attributable to a neutral tryptophan radical,[19] which in azurin can only be W48$^{\bullet}$. Since the p$K_a$ of the radical cation (eq 3) should be well below ∼4 (the value for the free amino acid)[20,21] in the hydrophobic core of the protein $\beta$-barrel (Figure 1B), proton release to the aqueous medium (eq 4) is expected.[22] The EPR spectrum of a frozen solution of irradiated $[Re(CO)_3$-(phen)(H107)]$^+AzZn^{2+}$ (in a Y72F variant of (H107)Az, leaving only one tyrosine in the protein) is shown in Figure 2A; the $g = 2.0042$ signal with partially resolved hyperfine splitting is the signature of a neutral tyrosyl radical with moderate dihedral angles (45° or more) for $C_{ring}-C_\beta-H_\beta$ ($Y108^{\bullet}$).[23]

**Scheme 1**

$$[Re(H83)]^+Az(W48)M^{2+} \xrightleftharpoons{h\nu} *[Re(H83)]^+Az(W48)M^{2+} \quad (1)$$

$$[Re(H83)]^+Az(W48)M^{2+} \xrightarrow{[Co(NH_3)_5Cl]^{2+}} [Re(H83)]^{2+}Az(W48)M^{2+} \quad (2)$$

$$[Re(H83)]^{2+}Az(W48)M^{2+} \xrightarrow{ET} [Re(H83)]^+Az(W48^{\bullet+})M^{2+} \quad (3)$$

$$[Re(H83)]^+Az(W48^{\bullet+})M^{2+} \xrightarrow{-H^+} [Re(H83)]^+Az(W48^{\bullet})M^{2+} \quad (4)$$

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(15) Other abbreviations: PSII, photosystem II.; im, imidazole; EPR, electron paramagnetic resonance; NHE, normal hydrogen electrode; $P_i$, phosphate; bpy, 2,2′-bipyridine; Otf, trifluoromethanesulfonate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

(16) Rhenium binding to surface histidines was achieved by reacting the proteins with aqueous $[Re(CO)_3(L)(H_2O)](Otf)$ (L = phen, 4,7-$Me_2$phen. Sullivan, B. P.; Meyer, T. J. *J. Chem. Soc., Chem. Commun.* **1984**, 1244−1245. Connick, W. B.; Di Bilio, A. J.; Schaefer, W. P.; Gray, H. B. *Acta Crystallogr. C* **1999**, *C55*, 913−916); the general procedure is the same as that employed in the preparation of Ru−plastocyanin (Di Bilio, A. J.; Dennison, C.; Gray, H. B.; Ramirez, B. E.; Sykes, A. G. *J. Am. Chem. Soc.* **1998**, *120*, 7551−7556). Details are given in the Supporting Information. Crystals of $[Re(CO)_3(4,7\text{-}Me_2phen)(H107)]^+AzCu^{2+}$ (space group *P*1, cell dimensions $a = 35.00$ Å, $b = 42.87$ Å, $c = 48.51$ Å, $\alpha = 80.52°$, $\beta = 77.69°$, $\gamma = 66.97°$, two molecules per asymmetric unit) grew from 2 $\mu$L drops containing 26 mg/mL $[Re(CO)_3(4,7\text{-}Me_2phen)(H107)]^+AzCu^{2+}$ in 25 mM HEPES pH 7.5, equilibrated against a 500 $\mu$L reservoir containing 20% PEG molecular weight 8000, and 100 mM im pH 8.0. Diffraction data (30.0−1.8 Å resolution, 84.2% complete, $R_{sym} = \Sigma\Sigma_j|I_j - \langle I\rangle|/\Sigma\Sigma_j I_j = 6.1\%$; overall signal-to-noise ratio = $I/\sigma I = 20.4$) were collected on an R-Axis IV image plate mounted on a Rigaku X-ray generator (1.5418 Å Cu K$\alpha$ radiation) and processed with DENZO (Otwinowski, Z.; Minor, W. *Methods Enzymol.* **1997**, *276*, 307−326). The structure of $[Re(CO)_3(4,7\text{-}Me_2phen)(H107)]^+AzCu^{2+}$ was determined by molecular replacement with EPMR (Kissinger, C. R.; Gehlhaar, D. K.; Fogel, D. B. *Acta Crystallogr.* **1999**, *D*55, 484−491) using a probe derived from the structure of $[Ru(bpy)_2(im)(H83)]^{2+}AzCu^{2+}$ (Faham, S.; Day, M. W.; Connick, W. B.; Crane, B. R.; Di Bilio, A. J.; Schaefer, W. P.; Rees, D. R.; Gray, H. B. *Acta Crystallogr.* **1999**, *D55*, 379−385; PDB code: 1BEX). Rigid-body, simulated-annealing, positional and thermal factor refinement with CNS (Brunger, A. T.; Adams, P. D.; Clore, G. M.; DeLano, W. L.; Gros, P.; Grosse-Kunstleve, R. W.; Jiang, J. S.; Kuszewski, J.; Nilges, M.; Pannu, N. S.; Read, R. J.; Rice, L. M.; Simonson, T.; Warren, G. L. *Acta Crystallogr.* **1998**, *D*54, 905−921), amidst rounds of manual rebuilding, $[Re(CO)_3(4,7\text{-}Me_2phen)]^+$ incorporation, and water placement with XFIT (McRee, D. E. *J. Mol. Graphics* **1992**, *10*, 44−46) produced the final model (1.8 Å resolution, $R_{factor} = 23.8\%$; $R_{free} = 25.9\%$; against 8.0% of the free reflections removed from refinement; rmsd angles = 1.4°, rmsd bonds = 0.008 Å). PDB code: 1I53. All residues have favored backbone dihedral angles. Stereochemical restraints were removed from the copper ligand bonds in the later stages of refinement. The structure of $[Re(CO)_3(phen)(H83)]^+AzCu^{2+}$ also has been determined (1.6 Å resolution: Crane, B. R.; Di Bilio, A. J.; Winkler, J. R.; Gray, H. B., in preparation).

(17) The method employs $[Co(NH_3)_5Cl]^{2+}$ as an irreversible oxidative quencher; previous work includes generation of porphyrin radicals and high-valent hemes (compounds I and II) in horseradish peroxidase (Berglund, J.; Pascher, T.; Winkler, J. R.; Gray, H. B. *J. Am. Chem. Soc.* **1997**, *119*, 2464−2469) and photoinduced oxidation of substituted tyrosine-Ru model compounds (Sun, L.; Burkitt, M.; Tamm, M.; Raymond, M. K.; Abrahamsson, M.; LeGourriérec, D.; Frapart, Y.; Magnuson, A.; Kenéz, P. H.; Brandt, P.; Tran, A.; Hammarström, L.; Styring, S.; Åkermark, B. *J. Am. Chem. Soc.* **1999**, *121*, 6834−6842). The $[Re(CO)_3(phen)(im)]^{2+/+}$ reduction potential is 1.85 V vs SCE in acetonitrile solution (Connick, W. B.; Di Bilio, A. J.; Hill, M. G.; Winkler, J. R.; Gray, H. B. *Inorg. Chim. Acta* **1995**, *240*, 169−173).

(18) EPR spectra were recorded with an X-band Bruker EMX spectrometer equipped with a standard $TE_{102}$ cavity. The magnetic field was calibrated against degassed 1% perylene in $H_2SO_4$; a built-in frequency counter provided accurate resonant frequency values. Variable temperature experiments were performed with an Oxford (ES9000) helium cryostat, whereas a finger Dewar was used in experiments at 77 K. Deaerated samples containing 0.5−2 mM Re-protein and 5−12 mM $[Co(NH_3)_5Cl]^{2+}$ in pH 7.0 $KP_i$ buffer (held in standard EPR quartz tubes) were irradiated while cooling in an unsilvered Dewar filled with liquid nitrogen; the excitation source was a focused beam from a xenon lamp (suitable filters were used to remove light with $\lambda < 310$ nm) or a YAG laser (355 nm, collimated beam) operating at 10 Hz. Photolysis occurred in fluid rather than frozen solution, as no EPR signals were observed from irradiated frozen samples.

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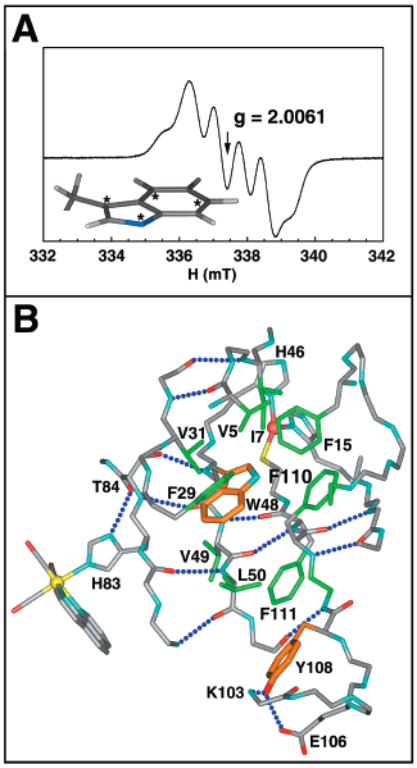


**Figure 1.** (A) EPR spectrum of a frozen glass (200 K) of irradiated $[Re(CO)_3(phen)(H83)]^+AzZn^{2+}/[Co(NH_3)_5Cl]^{2+}$ in $KP_i$/ethylene glycol; the spectrum does not change between 20 and 200 K, except for small line width variations (spectrometer settings: $\nu = 9.4739$ GHz, modulation frequency 100 kHz, modulation amplitude 3.0 G, microwave power 1.3 mW, time constant = 2.56 ms, conversion time = 10.24 ms, 40 scans). Inset: sites (asterisks) of high electron density in W• (Lendzian, F.; Sahlin, M.; MacMillan, F.; Bittl, R.; Fiege, R.; Pötsch, S.; Sjöberg, B.-M.; Gräslund, A.; Lubitz, W.; Lassmann, G. *J. Am. Chem. Soc.* **1996**, *118*, 8111−8120). (B) Environment of W48 and Y108 in $[Re(CO)_3(phen)(H83)]^+AzCu^{2+}$. Covalent and hydrogen bonds (dotted lines) of the azurin $\beta$-barrel link $[Re(CO)_3(phen)(H83)]^+$ to W48 and Y108. There are no ionizable residues or solvent molecules in the vicinity of W48, and the indole −N−H does not form a hydrogen bond with any protein residue. W48 contacts I7, I20, V31, L50, F110, and L125 (shown in green); the Y108 hydroxyl accepts a hydrogen bond from the K103 peptide nitrogen and donates a hydrogen bond to an E106 carboxyl oxygen.

Both K103 and E106 form hydrogen bonds to the Y108 hydroxyl in $[Re(CO)_3(phen)(H83)]^+AzCu^{2+}$ (Figure 1B),[16] thereby partially shielding the tyrosine side chain from solvent (17.9 Å$^2$ exposed surface area). In $[Re(CO)_3(4,7\text{-}Me_2phen)(H107)]^+AzCu^{2+}$, however, the E106 side chain swivels away from Y108 (a change in $\chi^2$ from trans (∼180°) to gauche$^+$ (∼60°)); this movement in the direction of the rhenium complex (Figure 2B) further exposes the Y108 hydroxyl to solvent (32.4 Å$^2$ exposed surface area). The change in E106 conformation most likely is due to the Q107H mutation and the Re positive charge rather than crystal packing, because both azurin molecules in the asymmetric unit of the crystal lattice are similarly structured. The increased exposure of Y108 in the H107 protein facilitates deprotonation/oxidation of the side chain hydroxyl.

The most striking finding is that W48•(H83) does not oxidize Y108(H83) on the time scale of our freeze-trapping experiments.

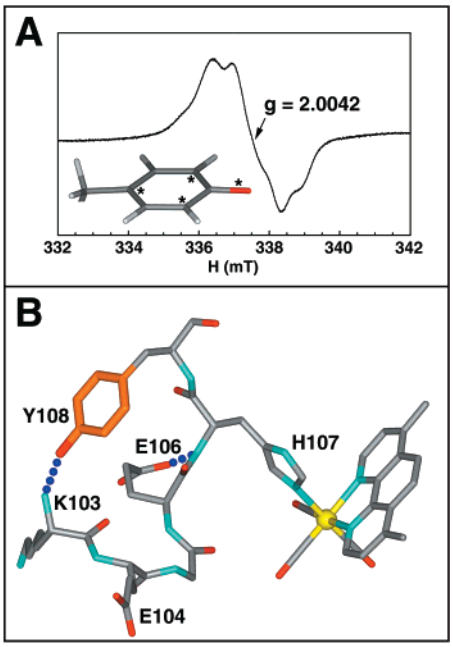


**Figure 2.** (A) EPR spectrum of a frozen solution (77 K) of irradiated $[Re(CO)_3(phen)(H107)]^+(Y72F)AzZn^{2+}/[Co(NH_3)_5Cl]^{2+}$ in pH 7.0 $KP_i$ (spectrometer settings: $\nu = 9.4671$ GHz, modulation frequency 100 kHz, modulation amplitude 2.0 G, microwave power = 0.0063 mW, time constant = 2.56 ms; conversion time = 10.24 ms, 40 scans). The EPR spectrum of irradiated $[Re(CO)_3(phen)(H107)]^+AzZn^{2+}/[Co(NH_3)_5Cl]^{2+}$ in frozen solution is virtually identical with that shown. Inset: sites (asterisks) of high electron density in Y• (Gräslund, A.; Sahlin, M. *Annu. Rev. Biophys. Biomol.* **1996**, *25*, 259−286). **(B)** Environment of Y108 in $[Re(CO)_3(4,7\text{-}Me_2phen)(H107)]^+AzCu^{2+}$. Y108 resides in a relatively hydrophilic environment near the molecular surface; the 108-hydroxyl accepts a hydrogen bond from the K103 peptide nitrogen, and the E106 carboxyl has swung away and is bonded to −H−N(H107).

One explanation is that W48• is the thermodynamic product of $[Re(H83)]^+AzM^{2+}$ oxidation, which would mean that the W•/W > Y•/Y order of reduction potentials in aqueous solution (pH 7)[24] is reversed in $[Re(H83)]^+AzM^{2+}$. Another possibility is that W48•(H83) is kinetically trapped at 200 K, which could occur if $k_{ET}$ (Y108 → W48•) were less than 0.1 s$^{-1}$ at room temperature. Rate estimates indicate that this ET reaction is not likely to be so slow; with $k_{ET}(max) = 2 \times 10^8$ s$^{-1}$ (108−48 distance:[16] 13 Å),[11,12,25] $k_{ET} < 0.1$ s$^{-1}$ requires that p$K_a$(Y108) be above 12 to inhibit generation of Y108• by deprotonation/oxidation; and $\Delta G°$(Y108/W48• (+H$^+$)→ Y108•$^+$/W48) would have to be greater than 0.5 eV ($\lambda = 1$ eV)[12] to disfavor a pathway involving initial formation of Y108•$^+$. On the basis of this analysis, it is likely that the W48•/W48 reduction potential is below that of Y108•/Y108 in the H83 protein.

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**Supporting Information Available:** Preparation and properties of Re-modified azurins; simulations of EPR spectra (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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